The role of apolipoprotein N-acyl transferase, Lnt, in the lipidation of Factor H binding protein of *Neisseria meningitidis* strain MC58 and its potential as a drug target.

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Running title Investigating the role of Lnt in *N. meningitidis*

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Abstract

Background and Purpose

The level of cell surface expression of the meningococcal vaccine antigen, Factor H binding protein (FHbp) varies between and within strains and this limits the breadth of strains that can be targeted by FHbp-based vaccines. The molecular pathway dictating expression of FHbp at the cell surface, including its lipidation, sorting to the outer membrane and export, and the potential regulation of this pathway have not been investigated until now. This knowledge will aid our evaluation of FHbp vaccines.

Experimental Approach

A meningococcal transposon library was screened by whole cell immuno-dot blotting using an anti-FHbp antibody to identify a mutant with reduced binding and the disrupted gene was determined.

Key Results

In a mutant with markedly reduced binding, the transposon was located in the *lnt* gene which encodes apolipoprotein N-acyl transferase, Lnt, responsible for the addition of the third fatty acid to apolipoproteins prior to their sorting to the outer membrane. We provide data indicating that in the Lnt mutant, FHbp is diacylated and its expression within the cell is reduced 10 fold, partly due to inhibition of transcription. Furthermore the Lnt mutant showed 64 fold and 16 fold increase in susceptibility to Rifampicin and Ciprofloxacin respectively.

Conclusion and Implications

We speculate that the inefficient sorting of diacylated FHbp in the meningococcus results in its accumulation in the periplasm inducing an envelope stress response to down-regulate its expression. We propose Lnt as a potential novel drug target for combination therapy with antibiotics.

Ligands	
Ceftriaxone	Erythromycin
Glucose	Penicillin G
Rifampicin	

These Tables of Links list key ligands in this article that are hyperlinked* to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al*., 2016), and are permanently archived in The Concise Guide to PHARMACOLOGY 2015/16 (Alexander *et al*., 2015).

Abbreviations

FHbp, Factor H binding protein,

SBA, serum bactericidal antibody

Lnt, apolipoprotein N-acyl transferase

Transposon, Tn

DNA uptake sequence, DUS

Mosaic End, ME

Hexahistidine, His

Tris buffered saline, TBS

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis, SDS-PAGE

Minimum Inhibitory Concentration, MIC

Lipoprotein Outer membrane Localisation apparatus, Lol

Diacylglyceryl transferase, Lgt

Lipoprotein signal peptidase, Lsp

Braun's lipoprotein, Lpp

Peptidoglycan, PGN

Whole cell, WC

Monoclonal antibody, Mab

Introduction

Neisseria meningitidis is a leading cause of bacterial meningitis (Beernink and Granoff, 2008). This organism is classified into 13 different serogroups depending on its capsular polysaccharide. Invasive meningococci typically express polysaccharides, A, B, C, W135, X or Y (Jolley *et al*., 2007). Effective protein-polysaccharide conjugate vaccines are available against serogroup A, C, W135 and Y meningococci (Snape and Pollard, 2005) however the capsular polysaccharide of serogroup B strains is poorly immunogenic making this type of vaccine ineffective (Yongye *et al*., 2008). Thus non-capsular, conserved, surface antigens such as the lipoprotein, Factor H binding protein (FHbp), have been tested for their ability to protect against organisms expressing the group B capsule.

Lipoproteins are a diverse class of multifunctional, membrane-associated molecules which constitute a significant fraction of the outer membrane of Gram-negative bacteria (Nakayama *et al*., 2012). Their diverse functions range from maintaining envelope architecture and stability to mediating host-pathogen interactions (Okuda and Tokuda, 2011, Zuckert, 2014, Nakayama *et al*., 2012). FHbp, as its name suggests, binds human factor H which enables the meningococcus to evade killing by human complement (Madico *et al*., 2006). Importantly immunisation with FHbp induces serum bactericidal antibody (SBA) responses that confer protection against the meningococcus (Borrow and Miller, 2006).

FHbp is lipidated by three palmitoyl fatty acids (Mascioni *et al*., 2010). Non-lipidated FHbp is part of the Bexsero vaccine (Novartis) which was licenced in Europe in 2013 (reviewed by McNeil *et al*., 2013). Fletcher *et al*., (2004) demonstrated the immunogenic potency of the lipid moiety of FHbp by directly comparing lipidated and non-lipidated versions in mice and

showing that the lipidated form elicited profoundly greater immunogenicity and breadth of protection compared with the non-lipidated form. Pfizer then developed a vaccine composed of 2 common variants of lipidated FHbp which was licenced in the US in 2014. However, it is uncertain whether, in the very young, these serogroup B vaccines can induce sufficiently robust, broad and sustained immune responses. It is also not clear in adolescents, (an age group clearly having a role in the carriage and transmission of meningococcal disease) whether these vaccines elicit sufficient breadth of coverage and potency of the immune response to interrupt transmission (McNeil *et al*., 2013). Limitations in the breadth of coverage could be explained by the fact that not all strains express FHbp and different strains express different variants of FHbp which furthermore are expressed at different levels (Biagini *et al*., 2016). Importantly Koeberling and co-workers demonstrated that a "critical threshold" of FHbp expression is required to elicit broad protective SBA responses (Koeberling *et al*., 2011).

Whilst the influence of environmental factors affecting FHbp expression levels, such as oxygen, temperature and iron availability has been established (Sanders *et al*., 2012, Oriente *et al*., 2010, Loh *et a*l., 2013), the molecular pathway for FHbp expression, including transport across the inner membrane followed by lipidation and sorting to the outer membrane and export to the cell surface, is unknown. This pathway inevitably requires a plethora of proteins whose genes may themselves be subject to regulation subsequently affecting FHbp expression levels. This study explores the molecular pathway governing FHbp expression which will ultimately improve our understanding of the variability in FHbp expression and aid us in our evaluation of FHbp-based vaccines.

Methods

Bacterial strains and culture conditions

N. meningitidis MC58, serogroup B:15:P1.7,16, ST-74; ET-5 strain was purchased from LGC Standards and L91543 serogroup C:2aP1.2, ST-11; ET-37 strain was kindly provided by Professor McFadden (University of Surrey). All meningococcal strains were grown on GC agar plates (Difco) containing Kellogg's glucose and iron supplements (Kellogg *et al*., 1963) in a moist atmosphere containing 5% CO_2, at 37°C or at 30°C for transformation experiments.

For each experiment, meningococcal cell suspensions were made by resuspending a loop of cells from a freshly grown overnight plate in PBS (137 mM NaCl, 16 mM phosphate, 2.7 mM KCl, pH 7.4) and adjusting to the required optical density at A_{600}. Serial dilutions of inocula were plated to verify consistency in colony forming unit (cfu) counts between strains.

Escherichia coli strain JM109 single use competent cells (Promega) were grown on Luria-Bertani (LB) agar or LB broth (Merck) at 37°C with shaking at 200 rpm.

Antibiotics were added at the following concentrations; Kanamycin, 30 and 60 μg/ml; Erythromycin, 300 and 0.3 μg/ml for *E. coli* and *N. meningitidis* respectively.

Molecular Methods for DNA manipulations

Genomic DNA was extracted from *N. meningitidis* using the Gentra Puregene Yeast/Bact Kit (Qiagen) and plasmid DNA was extracted from *E. coli* using the QiaPrep Spin kit (Qiagen). DNA samples were analysed by agarose gel electrophoresis and visualized by staining with

SYBR Safe (Invitrogen). Restriction enzymes (New England Biolabs), T4 DNA ligase (Promega), Antarctic Phosphatase (NEB) and T4 Polymerase (Thermo Scientific) were used according to the manufacturer's recommendations. PCRs were performed using HotStar HiFidelity polymerase kit (Qiagen) in a Perkin- MJ Research PTC-200 Peltier Thermal Cycler. Primers were purchased from Sigma and their sequences are listed in Table S1. PCR products and restriction digested DNA were purified using the PCR Mini Elute kit (Qiagen). *E. coli* was transformed by heat shock (Froger and Hall, 2007).

Construction of the Transposon library in strain MC58

Modification of EZ::Tn5<KAN-2> transposon to incorporate DUS

The transposon (Tn) from the EZ::Tn5<KAN-2> insertion kit (Epicentre), was modified to incorporate the DNA uptake sequence (DUS) known to facilitate the uptake of DNA in Neisseriaceae (Frye *et al*., 2013). This was achieved by PCR amplification of the EZ::Tn5<KAN-2> Tn using forward primer *Hin*dIII*kan2*for which incorporates a *Hin*dIII site and anneals immediately downstream of the 5' mosaic end (ME) of the Tn and the reverse primer *Eco*RIDUS*kan2*rev which incorporates an *Eco*RI site and DUS element and anneals downstream of the stop codon and upstream of the 3' ME.

The PCR product was cloned into the *Eco*RI, *Hind*III sites of plasmid pMOD$_{TM}$-2<MCS> (Epicentre). The ligation mixture was used to transform *E. coli* and transformants were selected by growth on Kanamycin. Clones were verified by PCR using primers PCRFP and PCRRP (Epicentre) and by sequencing (MWG Biotech). The resulting plasmid was designated pMOD$_{TM}$-2<Tn5KAN-2DUS>. Phosphorylated primers PCRFP and PCRRP

(Sigma) were used to amplify the region encompassed by and including the MEs from plasmid pMOD$_{TM}$-2<Tn5KAN-2DUS>.

In vitro transposition and transformation of strain MC58

1 µg of *Dra*I digested and purified genomic DNA of strain MC58 was mixed with 0.6 µg of phosphorylated PCR product, Ez-Tn5 reaction buffer and 4 U of transposase (Epicentre) in a total volume of 40 µl. The reaction was incubated at 37ºC for 2 hours then stopped according to the manufacturer's instructions. The DNA was purified and 3' overhangs were repaired by adding 5 U of T4 DNA polymerase to 1 µg of the DNA, 100 µM of each dNTP and T4 DNA polymerase buffer to a total volume of 50 µl. The reaction was incubated at 37ºC for 5 minutes followed by heat inactivation at 75ºC for 10 minutes. The DNA was purified and 6 U of T4 ligase and ligase buffer were added to the DNA to a total volume of 50 µl and the reaction was incubated at 21ºC for 2 hours. The DNA was again purified and 250 ng used to transform *N. meningitidis* as described by Zhang *et al*., (2010) with selection on LB-Kanamycin.

Construction of pGCC4S*fHbpHis*

Adopting the approach of Kurokawa *et al*., (2012), a truncated version of the FHbp protein, incorporating just the first 100 amino acids (from the cysteine at +1), fused to a His tag was expressed in *N. meningitidis* strains to differentiate between di- and tri-palmitoylated FHbp. The *fHbp* N-terminal region containing the N terminal signal peptide sequence which is predicted by the program DOLOP (Babu *et al*., 2006) to be MTRSKPVNRTAFCCLSLTTALILTAC, was PCR amplified from genomic DNA of strain MC58 with primer *BamfHbp*for which anneals upstream of the signal peptide and

incorporates a *Bam*HI site and primer *XhofHbp*rev which anneals 298 bp downstream of the signal peptide and incorporates a *Xho*I site to reduce the size of the translated product from 280 amino acids to 125 amino acids, prior to processing, and 100 amino acids after processing. The PCR product obtained was cloned into the *Bam*HI, *Xho*I sites of pET28b and transformed into *E. coli* with selection on Kanamycin. The resulting plasmid, pET28bSfHbp was verified by DNA sequencing. The SfHbp-His region was PCR amplified from plasmid pET28bS*fHbp* using primers *Pac*fHbpfor incorporating a *Pac*I site and *Pme*pET28brev incorporating a *Pme*I site. The PCR product was cloned into the *Pac*I, *Pme*I sites of *Neisseria* complementation vector, pGCC4 (Addgene) (Mehr and Seifert, 1998) and transformed into *E. coli* with selection on Erythromycin. The resulting plasmid, pGCC4S*fHbp*His was verified by DNA sequencing and the plasmid used to transform *N. meningitidis* strains as described by Zhang *et al*., (2010) with selection on Erythromycin. Transformant strains were verified by PCR then grown on GC agar containing 0.5 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) for expression studies.

Construction of the apolipoprotein N-acyl transferase, *lnt* complementation plasmid

The *lnt* gene of strain MC58 was cloned into the *Fse*I and *Pac*I sites of pGCC4 (Addgene). Briefly, primer *lntFse*Ifor incorporating an *Fse*I site and primer *lntPac*Irev incorporating a *Pac*I site were used to amplify a region from 158 nucleotides upstream of the start codon to 18 nucleotides downstream of the stop codon from genomic DNA of strain MC58. The PCR product obtained was cloned into the *Fse*I, *Pac*I sites of plasmid pGCC4. Cloning of *lnt* in the opposite direction to the *lacZ* promoter of pGCC4 allowed transcription from the native promoter of *lnt* and avoided over-expression of *lnt* by the *lacZ* promoter. Following transformation of *E. coli* with selection on Kanamycin, clones were verified by PCR and

sequencing then correct clones designated pGCC4*lnt*. The plasmid was linearized with *Nco*I which cuts pGCC4*lnt* uniquely in the Kanamycin resistance gene then used to transform strain MC58Lnt.

Primary antibodies

The following primary antibodies were used; mouse anti-FHbp-monoclonal antibody (Mab) JAR4 (National Institute for Biological Standards and Control), rabbit anti-RecA antibody (Abcam) and rabbit anti-His antibody (Santa Cruz Biotechnology).

Immuno-dot blotting to screen the Transposon library

Cell suspensions of the meningococcal strains were heat killed at 65ºC for 30 minutes and 5 µl were spotted onto a Nitrocellulose membrane (Thermo Scientific), dried then blocked in PBS containing 0.05% (v/v) Tween 20 (PBST) and 2% BSA (w/v) (Sigma) with gentle shaking for 1 hour. Three 2 minute washes in PBST were conducted then the membrane incubated in PBST containing 2% BSA (w/v) and 1 µg/ml of anti-FHbp antibody, JAR4, with gentle shaking for 3 to 4 hours. The washes were repeated and the membrane incubated in anti-mouse alkaline-phosphatase conjugated secondary antibody solution (Invitrogen) for 1 to 3 hours, washed as before then developed with 5-bromo-4-chloro-3-indolylphosphate, nitro-blue tetrazolium liquid substrate (Invitrogen).

Locating the Transposon in the mutant strain with altered binding to anti-FHbp antibody

In order to locate the Tn in the selected mutant strain and to verify the insertion of only one Tn in its genome, Ion Torrent genome sequencing was performed. The sequencing library was prepared using 0.5 µg of genomic DNA and the "fragmentation and library preparation kit" (New England Biolabs), according to the manufacturer's instructions. The adapter ligated library fragments were separated by gel electrophoresis followed by extraction of approximately 500 bp fragments from a 2% (w/v) E-gel (Life Technologies). The gel extracted DNA fragments were PCR amplified using 6 cycles as described in the "fragmentation and library preparation kit".

The size and concentration of the amplified library were checked using the BioAnalyser and a high sensitivity DNA kit (Agilent). The library concentration was adjusted to 20 pM and used for template preparation using the One Touch 2 system (OT2, Life Technology). The ion particles were enriched using an automatic system (OT2, Life Technology), loaded onto 314v2 chip and the sequencing was run using 400 bp sequencing kit and IonTorrent PGM with 850 flows. The reads were assembled using IonTorrent assembler plugin.
The contigs generated were analysed using CLC Genomics Workbench software v. 7. The contigs containing the Tn sequence were identified using BLASTn program running the extracted Tn sequence against the IonTorrent assembly. The contig containing the Tn sequence was further analysed using NCBI BLASTx and non-redundant amino acid sequence database to determine the genes flanking the Tn insertion site.

SDS-PAGE and Western immunoblotting

Whole cell (WC) lysates were prepared by mixing 600 µl of cell suspension, from each strain, standardised to A_{600} 1 with 300 µl 3X Laemmli sample buffer and heated to 95°C for 5 minutes. Cell lysate proteins were then separated by 12% (w/v) SDS-PAGE and transferred to PVDF or nitrocellulose membranes as appropriate. Membranes were then incubated with Tris-buffered saline, TBS (50 mM Tris, 150 mM NaCl, pH 7.5) containing 0.1% (v/v) Tween-20 (TBST) and 5% (w/v) non-fat dry milk for 2 hours at room temperature. Membranes were then incubated with either mouse anti-FHbp JAR4 (1 µg/ml) or rabbit anti-RecA (Abcam) primary antibody diluted (1:1000) in TBST containing 1% (w/v) non-fat dry milk overnight at 4°C. Membranes were washed for 60 minutes with TBST at room temperature and then incubated for 2 hours at room temperature with either sheep anti-mouse or donkey anti-rabbit HRP-linked secondary antibodies (GE Healthcare, UK), respectively. Membranes were washed with TBST for 30 minutes and specific protein bands were detected by enhanced chemiluminescence (GE Healthcare, UK). Band intensity was quantified using a GS-800 calibrated densitometer and Quantity One® 1-D analysis software v 4.6.2 (Bio-Rad).

Immunofluorescence microscopy

Bacterial suspensions from each strain were standardised to A_{600} 0.5 and 50 µl spotted onto 13 mm circular glass coverslips. Once dry, these were transferred to 24 well plates and cells fixed with 4% (v/v) paraformaldehyde for 20 minutes. The coverslips were washed in PBS then blocked with 500 µl PBS containing 1% (w/v) BSA for 30 minutes. The blocking agent was removed and 500 µl PBS containing 1% (w/v) BSA and 5 µg/ml of mouse anti-FHbp antibody, JAR4 added. This was incubated for two hours with agitation. The wells were washed three times in PBS then PBS with 1% (w/v) BSA containing 1:500 dilution Alexa

Fluor 555 labelled donkey anti-mouse IgG secondary antibody (Abcam) was added (to fluorescently label the FHbp-bound JAR4 antibody) and 1:500 dilution of FITC-labelled rabbit polyclonal IgG raised against WC *N. meningitidis* (Abcam) to detect meningococcal cells. After incubation for 1 hour with agitation, wells were washed as before, and the cover slips dipped in deionised water then mounted onto glass slides using fluoroshield mounting medium (Abcam). Fluorescence microscopy was performed using a Nikon Eclipse i80 and images captured using NIS-Elements Viewer (version BR 3.00).

Quantitative RT-PCR (qRT-PCR)

RNA was extracted from 1 ml cell suspensions of each strain standardised to A_{600} 0.65 (containing approximately 2 x 10^8 cells) using the RNeasy Mini kit (Qiagen) with enzymatic lysis and Proteinase K digestion. On column DNA digestion was performed using the RNase Free DNase set (Qiagen).

One µg of cDNA was synthesised using the QuantiTect reverse transcription kit (Qiagen) with the initial genomic wipe-out step included. qRT-PCR was performed in a 15 µl reaction mixture with Quantinova SYBR Green PCR Master Mix (Qiagen), 22.5 ng of cDNA and 0.7 µM of each primer (Sigma). For amplification of cDNA of *fHbp*, *fHbp* forward primer and *fHbp* reverse primer were used and for amplification of *recA*, *recA* forward primer and *recA* reverse primer were used. PCR was performed in a Prime Pro 48 Real Time PCR machine with the following thermocycling conditions; 95°C for 2 min followed by 40 cycles of 95 °C for 5s (denaturation) and 60°C for 10s (combined annealing/extension). Six biological replicates from 6 independent RNA extractions from each of the 3 strains were run in duplicate along with the corresponding no reverse transcriptase control for each of these samples and a no RNA control. Relative quantification of gene expression was performed

using the Comparative CT Method (ΔΔCt) (Livak and Schmittgen, 2002) whereby *fHbp* expression levels were normalized to the mean levels of control (*recA*) transcripts.

Antibiotic susceptibility assays by Microbroth Dilution

Meningococcal strains were compared for their susceptibility to a panel of antibiotics by the Microbroth Dilution method. For each antibiotic, the concentration range was centered around the Minimum Inhibitory Concentration (MIC) value according to the Clinical and Laboratory Standards Institute (CLSI). The antibiotics tested were Tunicamycin, Ceftriaxone, Penicillin G, Chloramphenicol, Sulfanilamide, Globomycin, Ciprofloxacin and Rifampicin.

Bacterial suspensions were standardised to A_{600} 0.4 then 5 µl added to 95 µl of Mueller Hinton broth containing doubling dilutions of antibiotic in a Thermo Scientific sterile 96 well plate (Nunclon Delta surface). Negative controls included no bacterial suspension and no antibiotic. For each MIC assay, all conditions were performed in triplicate and each assay was repeated at least 5 times. A gas permeable seal was placed over the plate and the plate incubated at 37°C in 5% CO_2 with gentle shaking for approximately 20 hours.

Statistical Analysis

The data and statistical analyses comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al*., 2015).

Data are shown as mean ± SEM. Multiple comparisons among groups were performed by one way ANOVA followed by Tukey's HSD test. A value of $p \leq 0.05$ was considered statistically

significant. Post-hoc tests were only run if F achieved $p<0.05$ and there was no significant variance in homogeneity. All statistical analysis tests were performed in IBM SPSS Statistics (v. 23).

Blinding and randomisation were not used as these were considered inappropriate for the *in vitro* experiments on bacteria conducted.

Results

Reduced binding to the FHbp-specific antibody following Tn insertion into *lnt*

A random Tn library of mutants was constructed in *N. meningitidis* strain MC58 known to express FHbp strongly at the cell surface (Newcombe *et al*., 2014). The first 100 mutant strains from the library were individually screened by WC immuno-dot blot using an anti-FHbp antibody, JAR4, as a probe for FHbp expression. The level of binding or reactivity with the antibody was compared with that of the parent strain MC58 and with that of strain L91543 known to express FHbp very weakly at the cell surface (Newcombe *et al*., 2014). Mutant 80 showed a marked reduction in reactivity to the antibody (Fig 1).

To identify which gene had been insertionally inactivated by the presence of the Tn in the mutant with reduced binding to the antibody, and to verify if only one Tn was present in this strain, Ion Torrent whole genome sequencing was performed. The single Tn identified was located at chromosomal position 745462, in the middle of gene NMB0713, annotated as *lnt,* which lies at chromosomal position 744476 to 746050. The mutant was designated MC58Lnt.

The *lnt* gene encodes the integral membrane protein, apolipoprotein N-acyl transferase (Lnt) which transfers the third fatty acid to the amide group of the N terminal conserved cysteine of apolipoproteins in bacteria (Chahales and Thanassi, 2015). In this mature form, lipoproteins that are destined for the outer membrane are then translocated by the lipoprotein outer membrane localisation (Lol) apparatus. Given that *lnt* was found to be the single disrupted gene in the mutant strain which was affected in its ability to bind an FHbp antibody, we predicted *lnt* is involved in FHbp acylation specifically in the addition of the third amide-linked palmitoyl fatty acid to the N terminal cysteine of FHbp.

FHbp is not fully mature in strain MC58Lnt

To test if Lnt is involved in FHbp acylation, truncated FHbp fused to a hexahistidine (His) reporter was expressed in strains MC58 and MC58Lnt using the approach of Kurokawa *et al*., (2012) and LoVullo *et al*., (2015) to generate sufficiently small recombinant lipoprotein to differentiate between diacylated and triacylated forms after separation by SDS-PAGE and detection by Western immunoblotting. Genomic DNA encoding the first 125 amino acid residues of FHbp, including the N terminal signal peptide, was cloned into pET28b to fuse this sequence to a C terminal His tag then the fusion construct was cloned into Neisseria vector, pGCC4 (Mehr and Siefert, 1998) for transformation into strains MC58 and MC58Lnt. The preprolipoprotein would be cleaved to yield a lipoprotein of 106 amino acids including the His tag, with a MW of 11.4 KDa as calculated by Expasy (Gasteiger *et al*., 2005) (not accounting for the attached fatty acids).

WC extracts from each strain were separated by SDS-PAGE and immunoblotted with anti-His antibody. As shown in Fig 2, the mobility of the reporter protein was greater in the

MC58Lnt transformant (lane 4) than in the wild type transformant (lane 3). The increase in mobility of the lipoprotein of the mutant strain was consistent with the expected change in acylation state at the N terminus, as such increased mobility has been shown by Mass Spectrometry to equate to the loss of a fatty acid (Kurokawa *et al*., 2012). Thus it appears that FHbp is diacylated in the Lnt mutant strain.

Disruption of Lnt strongly reduces FHbp cell surface expression

To confirm if the marked reduction in binding of whole cells of strain MC58Lnt to JAR4 (as shown by immuno-dot blotting) was due solely to the disruption of *lnt* in this strain, the mutant strain was complemented with an intact copy of the *lnt* gene from strain MC58. The *lnt* gene was cloned into vector pGCC4 (Mehr and Seifert 1998) such that expression was under the control of its own putative promoter to generate native levels of Lnt and used to transform strain MC58Lnt. Transformants were verified by PCR and sequencing and designated MC58LntC.

The expression of FHbp on the cell surface of individual cells of the strains was compared by immunofluorescence microscopy using JAR4. Cells of MC58Lnt showed very low levels of expression compared with that of the parent strain and cells of strain MC58LntC showed restored levels like that of the wild type (Fig 3). We concluded the disruption in Lnt alone was responsible for the poor expression of FHbp at the cell surface.

To further assess the level of surface expression of FHbp in strain MC58Lnt, its binding to JAR4 was compared with another meningococcal strain, L91543, known to express FHbp of the same variant 1 group (Masignani *et al*., 2003) and the same subfamily B (Beernink and Granoff, 2009; Fletcher *et al*., 2004), as strain MC58, with nucleotide and amino acid

identities of 95% and 93% respectively (Karlyshev *et al*., 2015), but very poorly at the cell surface (Newcombe *et al*., 2014). An immuno-dot blot using JAR4 was performed from standardised cell suspensions. Both strains MC58Lnt and L91543 showed weak reactivity to JAR4 but the level of reactivity was significantly lower for L91543 ($p \leq 0.0001$) compared with MC58Lnt (Fig 4). Confirming our initial immuno-dot blot and our immunofluorescence microscopy results, the level of antibody binding of strain MC58Lnt was significantly lower compared to the parent strain ($p \leq 0.0001$) and was restored in the complemented mutant strain. Together the results confirm that strain MC58Lnt expresses markedly reduced levels of FHbp at the cell surface compared to the parent strain and importantly reveal this strain expresses a higher level of FHbp at the cell surface compared with strain L91543 (Fig 4). This finding suggests that although FHbp is diacylated in strain MC58Lnt, it still sorts to the outer membrane to be exported to the cell surface although the quantity of the exported apo-(diacylated) form of FHbp is low.

Disruption of Lnt causes major reduction in total cellular levels of FHbp

To test if the low level of FHbp at the cell surface in strain MC58Lnt is due to inefficiency in the sorting of the apo-form of FHbp to the outer membrane we initially tested FHbp expression in WC extracts of the mutant strain before proceeding to investigate its localisation in the inner or outer membrane.

WC extracts of strains MC58, MC58Lnt and MC58LntC were fractionated by SDS-PAGE and immunoblotted with JAR4 (Fig 5). Surprisingly MC58Lnt showed a significant 10 fold reduction in band intensity ($p \leq 0.0001$) and this was restored in strain MC58LntC. A

consistent level of expression of the house-keeping protein RecA (Loh *et al*., 2013) was observed between the 3 strains.

The reduction in ability of the diacylated FHbp to bind JAR4 is unlikely due to loss of the fatty acid affecting conformation of the epitope known to bind this antibody (previously characterised by Beernink and Granoff, (2009)), since recombinant, non-acylated FHbp (lacking the signal peptide) strongly binds this antibody (data not shown). Thus we concluded that the mutation of Lnt in strain MC58 indeed results in a profound reduction in the cellular levels of FHbp.

Disruption of Lnt causes a reduction in *fHbp* transcription

To test if the marked reduction in FHbp in strain MC58Lnt could be attributed to downregulated transcription of *fHbp*, RNA was extracted from strains MC58Lnt, MC58 and MC58Lnt*C* and the level of transcription of *fHbp* compared between the strains and with the house-keeping gene, *recA*. Strain MC58Lnt showed a significant 53% reduction in the level of *fHbp* transcript relative to the parent strain ($p \leq 0.0001$) (Fig 6).

The Lnt mutant is more susceptibility to antibiotics

Since the disruption of Lnt likely affects a whole plethora of lipoproteins and lipoproteins are important for the integrity of the cell envelope, we predicted that the loss of Lnt would result in greater cell envelope permeability and therefore increased susceptibility of the mutant strain to antibiotics. The MICs of eight antibiotics were compared for strain MC58Lnt and the parent and complemented strains by the Microbroth Dilution method.

Strain MC58Lnt showed the following significant increases in antibiotic susceptibility; 64 fold to Rifampicin, 16 fold to Ciprofloxacin, 4 fold to both Globomycin and Sulphanilamide and 2 fold to Ceftriaxone, Penicillin G and Chloramphenicol (Table 1). There was no change in MIC for Tunicamycin.

Discussion

In this study we set out to identify genes involved in meningococcal cell surface expression of the vaccine antigen FHbp. A random Tn library was constructed in strain MC58 and individual Tn mutants were screened by WC immuno-dot blot using the anti-FHbp antibody, JAR4, as a probe. A mutant strain was identified with markedly reduced binding to the antibody. The Tn was located, by Ion Torrent whole genome shot-gun sequencing, in the *lnt* gene which encodes apolipoprotein N-acyl transferase, Lnt.

The activity of Lnt is the third step of the biosynthetic pathway for post-translational lipid modification of apolipoproteins that has been documented for *E. coli* (Kovacs-Simon *et al*., 2011) (Fig 7). This processing begins with translation in the cytoplasm of the precursor or preprolipoprotein which carries a cleavable N-terminal signal peptide possessing the conserved lipobox ([LVI][ASTVI][AGS][C]) at its C terminus. The preprolipoprotein is transported from the cytoplasm across the inner membrane usually by the general secretion pathway which is mediated by the Sec translocon (Fig 7). In the periplasm, the thiol group of the invariant cysteine residue of the lipobox is diacylated by a preprolipoprotein diacylglyceryl transferase, Lgt forming a prolipoprotein. Lipoprotein signal peptidase, Lsp, cleaves the signal peptide such that the di-acylated cysteine residue becomes the new N

terminal residue. Lnt then adds an additional amide-linked fatty acid to this residue generating a triacylated protein. The mature lipoprotein can then be recognised by Lol which transports it to the outer membrane, the default destination for lipoproteins. However if lipoproteins contain a "Lol avoidance" signal, (for example an aspartic acid at the +2 residue in *E. coli* or a lysine and a serine at positions +3 and +4 respectively, in *Pseudomonas* spp (Narita and Tokuda, 2007, Seydel *et al*., 1999), they are retained and anchored at the inner membrane (Hara *et al*, 2003). In *E. coli* the Lol apparatus consists of LolCDE, an ABC transporter which releases mature lipoproteins from the inner membrane, LolA, a chaperone or carrier protein which shuttles lipoproteins from the inner membrane to the outer membrane and LolB, an outer membrane lipoprotein receptor which inserts the lipoprotein into the outer membrane (Narita and Tokuda, 2006) (Fig 7). Most outer membrane lipoproteins are thought to face into the periplasm however some are exposed on the outer surface to face extracellularly, such as FHbp.

The generally accepted model, based largely on experiments performed in *E. coli*, is that complete triacylation of lipoproteins by Lnt is essential in Gram-negative bacteria, as it permits the LolCDE-dependent release of lipoproteins from the inner membrane for their sorting to the outer membrane (Fukuda *et al*., 2002, Robichon *et al*., 2005, Zuckert *et al*., 2014). Experiments using a conditionally lethal *E. coli* Lnt mutant showed that loss of Lnt led to incomplete maturation of lipoproteins and the retention in the inner membrane of those normally destined for the outer membrane, in particular, the retention of the highly abundant Braun's lipoprotein, Lpp, which subsequently cross-linked with peptidoglycan (PGN) causing lethality (Robichon *et al*., 2005, Yakushi *et al*., 1997).

In our study, firstly we show that unlike in *E. coli*, Lnt is not essential in *N. meningitidis*. This supports a recent study by LoVullo *et al*., (2015) who challenged the above model. LoVullo

and coworkers demonstrated Lnt was not essential in several Gram-negative bacteria they investigated including *Francisella novicida*, *Francisella tularensis* and *Neisseria gonorrhoeae* (LoVullo *et al*., 2015).

We confirmed the role of Lnt in adding a fatty acid to FHbp, by adopting the approach of Kurokawa *et al*., (2012) and LoVullo *et al*., (2015) to differentiate diacylated from triacylated lipoproteins. A truncated version of FHbp consisting of the N-terminal portion of FHbp (including its upstream signal peptide) fused to a His-tag was expressed in the wild type and Lnt mutant strain. We could infer from the mobility differences observed upon SDS-PAGE fractionation and Western immunoblotting that FHbp was diacylated in strain MC58Lnt (Fig 2).

We confirmed that the disruption of Lnt was directly responsible for the large reduction in binding of whole cells to JAR4, shown by immuno-dot blotting, by complementing strain MC58Lnt with an intact copy of the *lnt* gene. Immunofluorescence microscopy of meningococcal cells with JAR4 showed a low level of FHbp expression in the Lnt mutant and a restored level of expression in the complemented strain, like that of the wild type (Fig 3).

The level of expression of FHbp of strain MC58Lnt was assessed relative to strain L91543 which is known to express FHbp at the cell surface very poorly (Newcombe *et al*., 2012). Strain MC58Lnt showed a significantly higher level of reactivity to JAR4 compared to strain L91543 in WC immuno-dot blot studies (Fig 4). This result suggests that *N. meningitidis* has the ability to sort diacylated lipoproteins to the outer membrane prior to export to the cell surface although the quantity of exported FHbp was low.

Together our findings agree with those of LoVullo and co-workers (2015) who demonstrated that mutation of Lnt in *F. tularensis* resulted in lipoprotein Tul4 (LpnA) shifting from a triacylated form to a diacylated form and that this lipoprotein as well as others still sorted to the outer membrane.

LoVullo *et al*., (2015) showed that the Lol system of *F. tularensis* lacks a gene for LolE, which in *E. coli* forms a heterodimer with LolC to form the membrane component of the ABC transporter complex. They showed that the absence of *lolE* is not unique to *Francisella* but instead was represented by more than half of the bacterial genomes they analysed (LoVullo *et al*., 2015). Based on protein sequence analysis, LoVullo *et al* (2015) concluded that the single LolC present in bacteria such as *Francisella* spp contains features found in both LolC and LolE proteins. This suggests that the single LolC is a hybrid protein, which they named LolF. LoVullo *et al*., (2015) proposed that a homodimer formed by LolF enables the LolFD transporter complex of *Francisella* to recognise diacylated as well as triacylated lipoproteins and to transfer either type of substrate to LolA for sorting to the outer membrane (Fig 7). To test if their hypothesis could be extended to another Gram-negative bacterium, *Neisseria gonorrhoeae*, which has the same LolFD genomic organisation as found in *Francisella*, LoVullo *et al.*, (2015) demonstrated that viable Δ*lnt* mutants could be constructed in this organism, with proper lipoprotein-dependent functionality. LoVullo *et al*., (2015) proposed that the LolFD transporter may have a looser specificity for acyl chains and can recognise both with almost equal affinity in contrast to the LolCDE transporter of *E. coli* (Narita and Takuda, 2011). Narita and Takuda (2011) demonstrated that LolCDE of *E. coli* does have some affinity for apolipoproteins but this is very low, and required over expression of LolCDE for apolipoproteins to be released from the inner membrane and sorted to the outer membrane in their Lnt null mutant strain (lacking Lpp or the transpeptidases that cross link Lpp to PGN).

N. meningitidis has the same LolFD genomic organisation as that of *N. gonorrhoeae* and *F. tularensis* however given the low level of FHbp observed at the cell surface of strain MC58Lnt, we questioned if the LolFD transporter in this strain had low affinity for apolipoproteins resulting in inefficient sorting to the outer membrane and thus a low level of export to the cell surface. To test this, we investigated FHbp expression initially in WC extracts prior to assessing the compartmentalisation of FHbp in the inner and outer membrane. Surprisingly the total cellular level of FHbp expression of the mutant strain was reduced 10 fold compared to that of the parent and complemented strain (Fig 5). This finding suggests that transcription or translation of the partially acylated FHbp was being affected.

We investigated *fHbp* transcription by qRT-PCR in the Lnt mutant and showed that *fHbp* transcription was significantly downregulated by over 50% in strain MC58Lnt compared to the parent strain (Fig 6). However given the 10 fold decrease in FHbp levels in WC extracts, we predict that translation of FHbp is also downregulated or proteolysis of the apo-form of FHbp is induced or both.

To combat the problem of envelope stress caused by altered proteins translocated from the cytoplasm to the inner membrane, Gram-negative bacteria employ two major responses, one mediated by transcription factor σ^E and the other by Cpx to down regulate and degrade these proteins Whilst the σ^E response senses and mediates adjustments to changes in the biogenesis of proteins in the periplasm specifically outer membrane proteins (Ruiz *et al*., 2005), the Cpx response is activated by the over-expression of proteins or misfolded proteins that aggregate at the inner membrane (MacRitchie *et al*., 2008). Both responses result in the activation of proteases to degrade these altered proteins and both can upregulate small RNAs (sRNA) to downregulate the synthesis of these proteins by interfering with their transcription or translation (Walsh *et al*., 2003, Barchinger and Ades, 2013, Lima *et al*., 2013, Zhang *et a*l.,

2003, Raivio *et al*., 2013). For example, σ^E regulated sRNA, MicL specifically targets Lpp of *E. coli* by decreasing *lpp* translation and accelerating the degradation of *lpp* mRNA (Guo *et al*., 2014). There is a growing body of evidence that σ^E regulated pathways are adopted by the meningococcus (Huis in 't Veld *et al*., 2011, Hopman *et al*., 2010, Fagnocchi *et al.,* 2015).

Our study led us to propose the following model. The meningococcus can be grouped with *F. tularensis* and *N. gonorrhoeae*, and likely all other organisms that possess the LolFD transporter and can sort both di- and triacylated lipoproteins (LoVullo *et al*., 2011) negating the essentiality of Lnt however meningococcal LolFD has a higher affinity for triacylated lipoproteins than diacylated lipoproteins (Fig 7). We speculate that in strain MC58Lnt, the inefficient sorting of partially acylated lipoproteins such as the apo-form of FHbp to the outer membrane (indicated from its strongly reduced surface expression) results in their accumulation in the periplasm inducing envelope stress responses to downregulate their expression and promote their degradation. Comparative quantification of total cellular apolipoprotein levels in Lnt mutants of other Gram-negative bacteria (with that of their corresponding parent strains) will provide insight into whether apolipoproteins of these bacteria are also downregulated or proteolysed, due to the loss of Lnt, and whether our model therefore applies to other microorganisms. It is also important to note that the specific fate of apolipoproteins may vary if LolFD has different affinities for different apolipoproteins perhaps depending on their protein moiety, as has been suggested for LolCDE (Narita and Tokuda, 2011) or on their lipid moiety, or both.

Despite differences in the efficiency of sorting of diacylated lipoproteins in Gram-negative bacteria possessing the LolFD apparatus, the important question we now ask is whether these bacteria have the ability to naturally generate both di- and triacylated lipoproteins by regulation of Lnt. Whilst the lipid moieties of bacterial lipoproteins play an important

structural role by anchoring the lipoprotein to the cell wall, during infection lipoproteins can dissociate from the bacterium freeing the exposed lipid moieties to exert their immuno-modulatory activities (Luo *et al*., 2016). Diacylated and triacylated lipoproteins induce cell signalling by interacting with two different Toll-like receptor 2 (TLR2) heterodimers on antigen presenting cells. Specifically diacylated lipoproteins signal via TLR2/TLR6 heterodimers (Kang *et al*., 2009) and triacylated lipoproteins signal via TLR2/TLR1 heterodimers (Jin *et al*., 2007) and the latter was recently demonstrated for tripalmitoylated FHbp of Trumenba (Luo *et al*., 2016). This difference in signalling impacts on the ability of lipoproteins or lipopeptides to activate macrophages and subsequently to activate B cells (Metzger *et al*., 1995, Muhlradt *et al*., 1997, Muhlradt *et al*., 1998, Zeng *et al*., 2010). Thus the ability to generate two lipid moieties with different immunomodulatory activities and potencies would likely confer a fitness advantage to the bacterium in the host.

The intrinsic structure of the cell envelope of Gram-negative bacteria presents a significant barrier for the penetration of antibiotics (Graef *et al*., 2016). However in the meningococcal Lnt mutant strain we anticipated that the inefficient sorting of FHbp to the outer membrane and the potential inefficient sorting of other apolipoproteins would result in a structurally weaker outer membrane that would be more permeable to antibiotics. The antibiotics commonly used as initial therapy, particularly in adolescents, to treat invasive meningococcal infection are Penicillin G and third generation Cephalosporins, Cefotaxime and Ceftriaxone, and Chloramphenicol can be used in cases of Penicillin allergy (Nadel, 2016). The antibiotics often used for follow up treatment are Ceftrixone, Ciprofloxacin or Rifampicin (Nadel, 2016). We selected five of the above antibiotics as well as Tunicamycin, Sulfalinamide and Globomycin to compare the MICs for the Lnt mutant, parent and complemented strains.

The Lnt mutant was 64 fold more susceptible to Rifampicin, 16 fold more susceptible to Ciprofloxacin, 4 fold more susceptible to Globomycin and Sulfalinamide and 2 fold more susceptible to Penicillin G, Ceftriaxone and Chloramphenicol. The results support our prediction that the cell envelope of the Lnt mutant is weaker and more permeable to antibiotics. This highlights the importance of developing antimicrobials that target enzymes involved in constituting envelope integrity that could be used in combination with antibiotics. An inhibitor of Lnt would target all meningococcal strains since this protein is highly conserved between *N. meningitidis* strains as demonstrated by their 99% to 100% amino acid sequence identity across the entire protein (BLASTp analysis not shown). Importantly this enzyme is absent in humans.

Interestingly LoVullo *et al*., (2015) reported only small increases in susceptibility of their ΔLnt mutant of *N. gonorrhoeae* to Rifampicin, Globomycin, Polymixin B and Vancomycin amongst the antibiotics and chemical compounds they tested. The greater increase in sensitivity of the meningococcal Lnt mutant to antibiotics, shown in this study, compared with the small increase observed for the gonococcal ΔLnt mutant (LoVullo *et al*., 2015) supports the difference in the two models proposed for these organisms that LolFD of *N. gonorrhoaea* (as well as *F. tularensis*) sorts di- and triacylated lipoproteins efficiently maintaining the integrity of the outer membrane, in contrast LolFD of *N. meningitidis* appears to have a lower affinity for diacylated lipoproteins compared to triacylated lipoproteins and downregulates their expression resulting in a weaker outer membrane which is more permeable to antibiotics.

To conclude, from this study we show that for the meningococcus Lnt is a promising drug target and for the gonococcus we highlight the importance of targeting enzymes that act earlier in the lipoprotein processing pathway, such as Lgt, to prevent any sorting of diacylated

lipoproteins to the cell surface. Our study is very timely given the continuing rise in antibiotic resistance in both *N. meningitidis* and *N. gonorrhoeae* and the ever increasing need to develop new antimicrobials against these organisms (Zapun *et al*., 2016).

Author contributions

Griffin R devised the project and supervised da Silva RAG to construct the Tn library of mutants and conduct the qRT-PCR experiments, the MIC assays, generate the recombinant SFHbpHis strains and characterise these strains by Western analysis. Griffin R complemented the mutant strain and performed the immuno-dot blot assays and Western immunoblots with anti-FHbp antibody and anti-RecA antibody. Griffin R wrote the manuscript.

Churchward CP performed the immunofluorescence microscopy work.

Karlyshev AV conducted the Ion Torrent Sequencing.

Eleftheriadou O assisted with the qRT-PCR analysis and Western immunoblots.

Snabaitis AK provided reagents and equipment.

Longman MR gave advice for the Western immunoblots

Ryan A provided lab space, reagents, support and advice throughout this project.

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Conflicts of interest None

References

Alexander SP, Kelly E, Marrion N, Peters JA, Benson HE, Faccenda E, *et al*. (2015). The Concise Guide to PHARMACOLOGY 2015/16: Overview. *Br J Pharmacol.* **172**: 5729-43

Babu, MM, Priya, ML, Selvan, AT, Madera, M, Gough, J, Aravind, L, *et al*. (2006). A database of bacterial lipoproteins (DOLOP) with functional assignments to predicted lipoproteins. *J Bacteriol* **188**: 2761-73

Barchinger, SE, Ades, SE (2013). Regulated proteolysis: control of the *Escherichia coli* sigma(E)-dependent cell envelope stress response. *Subcell Biochem* **66**: 129-60

Beernink, PT, Granoff, DM (2009). The modular architecture of meningococcal factor H-binding protein. *Microbiology* **155**: 2873-83

Beernink, PT, Granoff, DM (2008). Bactericidal antibody responses induced by meningococcal recombinant chimeric factor H-binding protein vaccines. *Infect Immun* **76**: 2568-75

Biagini, M, Spinsanti, M, De Angelis, G, Tomei, S, Ferlenghi, I, Scarselli, M, *et al.* (2016). Expression of factor H binding protein in meningococcal strains can vary at least 15-fold and is genetically determined. *Proc Natl Acad Sci USA* **113**: 2714-9

Borrow, R, Miller, E (2006). Long-term protection in children with meningococcal C conjugate vaccination: lessons learned. *Expert Rev Vaccines* **5**: 851-7

Chahales, P, Thanassi, DG (2015). A more flexible lipoprotein sorting pathway. *J Bacteriol* **197**: 1702-4

Curtis MJ, Bond RA, Spina D, Ahluwalia A, Alexander SPA, Giembycz MA *et al. (*2015*).* Experimental design and analysis and their reporting: new guidance for publication in BJP. *Br J Pharmacol* **172***:* 3461–71

Fagnocchi L, Bottini S, Golfieri G, Fantappiè L, Ferlicca F, Antunes A *et al*. (2015) Global transcriptome analysis reveals small RNAs affecting Neisseria meningitidis bacteremia. *PLoS One* **10**: 1-22

Fletcher, LD, Bernfield, L, Barniak, V, Farley, JE, Howell, A, Knauf, M, *et al.* (2004). Vaccine potential of the *Neisseria meningitidis* 2086 lipoprotein. *Infect Immun* **72**: 2088-100.

Froger, A, Hall, JE (2007). Transformation of plasmid DNA into *E. coli* using the heat shock method. *J Vis Exp* **6**: 253.

Frye, SA, Nilsen, M, Tonjum, T & Ambur, OH (2013). Dialects of the DNA uptake sequence in Neisseriaceae. *PLoS Genet* **9**: 1-13

Fukuda, A, Matsuyama, S, Hara, T, Nakayama, J, Nagasawa, H & Tokuda, H (2002). Aminoacylation of the N-terminal cysteine is essential for Lol-dependent release of lipoproteins from membranes but does not depend on lipoprotein sorting signals. *J Biol Chem* **277**: 43512-8

Gasteiger E, Hoogland C, Gattiker A, Duvaud S, Wilkins MR, Appel RD *et al.* (2005) *Protein Identification and Analysis Tools on the ExPASy Server in The Proteomics Protocols Handbook. John M. Walker (ed). Humana Press* 571-607

Graef F, Gordon S, Lehr CM. (2016) Anti-infectives in Drug Delivery-Overcoming the Gram-Negative Bacterial Cell Envelope. *Curr Top Microbiol Immunol* 1-22

Guo, MS, Updegrove, TB, Gogol, EB, Shabalina, SA, Gross, CA & Storz, G (2014) A new sigmaE-dependent sRNA, combats envelope stress by repressing synthesis of Lpp, the major outer membrane lipoprotein. *Genes Dev* **28**: 1620-34

Hara, T, Matsuyama, S & Tokuda, H (2003). Mechanism underlying the inner membrane retention of *Escherichia coli* lipoproteins caused by Lol avoidance signals. *J Biol Chem* **278**: 40408-14

Hopman, CT, Speijer, D, van der Ende, A, Pannekoek, Y (2010). Identification of a novel anti-sigmaE factor in *Neisseria meningitidis*. *BMC Microbiol* **10**: 1-12

Huis in 't Veld, RA, Willemsen, AM, van Kampen, AH, Bradley, EJ, Baas, F, Pannekoek, Y, *et al.* (2011). Deep sequencing whole transcriptome exploration of the sigmaE regulon in *Neisseria meningitidis*. *PLoS One* **6**: 1-9

Jin MS, Kim SE, Heo JY, Lee ME, Kim HM, Paik SG, *et al*. (2007) Crystal structure of the TLR1-TLR2 heterodimer induced by binding of a tri-acylated lipopeptide. *Cell* **130**: 1071-82

Jolley, KA, Brehony, C & Maiden, MC (2007). Molecular typing of meningococci: recommendations for target choice and nomenclature. *FEMS Microbiol Rev* **31**: 89-96

Kang JY, Nan X, Jin MS, Youn SJ, Ryu YH, Mah S *et al*. (2009) Recognition of lipopeptide patterns by Toll-like receptor 2-Toll-like receptor 6 heterodimer. *Immunity* **18**: 873-84

Karlyshev, AV, Snyder, LA, McFadden, J & Griffin, R (2015). Insight into proteomic investigations of *Neisseria meningitidis* serogroup C strain L91543 from analysis of its genome sequence. *FEMS Microbiol Lett* **362**: 1-3

Kellogg DS Jr, Peacock WL Jr, Deacon WE, Brown L, Pirkle DI. (1963) *Neisseria gonorrhoeae*. I. Virulence genetically linked to clonal variation. *J. Bacteriol* **85:** 1274-9

Koeberling, O, Delany, I & Granoff, DM (2011). A critical threshold of meningococcal factor H binding protein expression is required for increased breadth of protective antibodies elicited by native outer membrane vesicle vaccines. *Clin Vaccine Immunol* **18**: 736-42

Kovacs-Simon, A, Titball, RW & Michell, SL (2011). Lipoproteins of bacterial pathogens. *Infect Immun* **79**: 548-61

Kurokawa, K, Kim, MS, Ichikawa, R, Ryu, KH, Dohmae, N, Nakayama, H, *et al.* (2012). Environment-mediated accumulation of diacyl lipoproteins over their triacyl counterparts in *Staphylococcus aureus*. *J Bacteriol* **194**: 3299-306

Lima, S, Guo, MS, Chaba, R, Gross, CA & Sauer, RT (2013). Dual molecular signals mediate the bacterial response to outer-membrane stress. *Science* **340**: 837-41

Livak KJ, Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**: 402-8

Loh, E, Kugelberg, E, Tracy, A, Zhang, Q, Gollan, B, Ewles, H, *et al.* (2013). Temperature triggers immune evasion by *Neisseria meningitidis*. *Nature* **502**: 237-40

LoVullo, ED, Wright, LF, Isabella, V, Huntley, JF & Pavelka, MS,Jr (2015). Revisiting the Gram-negative lipoprotein paradigm. *J Bacteriol* **197**: 1705-15

Luo Y, Friese OV, Runnels HA, Khandke L, Zlotnick G, Aulabaugh A, *et al*. (2016) The Dual Role of Lipids of the Lipoproteins in Trumenba, a Self-Adjuvanting Vaccine Against Meningococcal Meningitis B Disease. *AAPS J.* 1-14

MacRitchie, DM, Buelow, DR, Price, NL, Raivio, TL (2008). Two-component signalling and gram negative envelope stress response systems. *Adv Exp Med Biol* **631**: 80-110

Madico, G, Welsch, JA, Lewis, LA, McNaughton, A, Perlman, DH, Costello, CE, *et al.* (2006). The meningococcal vaccine candidate GNA1870 binds the complement regulatory protein factor H and enhances serum resistance. *J Immunol* **177**: 501-10

Mascioni, A, Moy, FJ, McNeil, LK, Murphy, E, Bentley, BE, Camarda, R, *et al.* (2010). NMR dynamics and antibody recognition of the meningococcal lipidated outer membrane protein LP2086 in micellar solution. *Biochim Biophys Acta* **1798**: 87-93

Masignani V, Comanducci M, Giuliani MM, Bambini S, Adu-Bobie J, Arico B, *et al*. (2003). Vaccination against *Neisseria meningitidis* using three variants of the lipoprotein GNA1870. *J Exp Med*. 2003 **97**: 789-99

McNeil, LK, Zagursky, RJ, Lin, SL, Murphy, E, Zlotnick, GW, Hoiseth, SK, *et al.* (2013). Role of factor H binding protein in *Neisseria meningitidis* virulence and its potential as a vaccine candidate to broadly protect against meningococcal disease. *Microbiol Mol Biol Rev* **77**: 234-52

Mehr, IJ, Seifert, HS (1998). Differential roles of homologous recombination pathways in *Neisseria gonorrhoeae* pilin antigenic variation, DNA transformation and DNA repair. *Mol Microbiol* **30**: 697-710

Metzger JW, Beck-Sickinger AG, Loleit M, Eckert M, Bessler WG, Jung G (1995) Synthetic S-(2,3-dihydroxypropyl)-cysteinyl peptides derived from the N-terminus of the cytochrome subunit of the photoreaction centre of *Rhodopseudomonas viridis* enhance murine splenocyte proliferation. *J Pept Sci* **1**: 184-90.

Mühlradt PF, Kiess M, Meyer H, Süssmuth R, Jung G. (1997) Isolation, structure elucidation, and synthesis of a macrophage stimulatory lipopeptide from *Mycoplasma fermentans* acting at picomolar concentration. *J Exp Med* **185**: 1951-8.

Mühlradt PF, Kiess M, Meyer H, Süssmuth R, Jung G. (1998) Structure and specific activity of macrophage-stimulating lipopeptides from *Mycoplasma hyorhinis*. *Infect Immun* **66**: 4804-10

Nadel S. (2016) Treatment of Meningococcal Disease. *J Adolesc Health*. **59**: S21-8

Nakayama, H, Kurokawa, K & Lee, BL (2012). Lipoproteins in bacteria: structures and biosynthetic pathways. *FEBS J* **279**: 4247-68

Narita, S, Tokuda, H (2006). An ABC transporter mediating the membrane detachment of bacterial lipoproteins depending on their sorting signals. *FEBS Lett* **580**: 1164-70

Narita, S, Tokuda, H (2007). Amino acids at positions 3 and 4 determine the membrane specificity of *Pseudomonas aeruginosa* lipoproteins. *J Biol Chem* **282**: 13372-8

Narita, S, Tokuda, H (2011). Overexpression of LolCDE allows deletion of the *Escherichia coli* gene encoding apolipoprotein N-acyltransferase. *J Bacteriol* **193**: 4832-40

Newcombe, J, Mendum, TA, Ren, CP & McFadden, J (2014). Identification of the immunoproteome of the meningococcus by cell surface immunoprecipitation and MS. *Microbiology* **160**: 429-38

Okuda, S, Tokuda, H (2011). Lipoprotein sorting in bacteria. *Annu Rev Microbiol* **65**: 239-59

Oriente, F, Scarlato, V & Delany, I (2010). Expression of factor H binding protein of meningococcus responds to oxygen limitation through a dedicated FNR-regulated promoter. *J Bacteriol* **192**: 691-701

Raivio, TL, Leblanc, SKD, Price, NL (2013). The *Escherichia coli* Cpx envelope stress response regulates genes of diverse function that impact antibiotic resistance and membrane integrity. *J Bacteriol* **195**: 2755-67

Robichon, C, Vidal-Ingigliardi, D & Pugsley, AP (2005). Depletion of apolipoprotein N-acyltransferase causes mislocalization of outer membrane lipoproteins in Escherichia coli. *J Biol Chem* **280**: 974-83

Ruiz, N, Silhavy, TJ (2005). Sensing external stress: watchdogs of the *Escherichia coli* cell envelope. *Curr. Opin. Microbiol.* **8:** 122-6

Sanders, H, Brehony, C, Maiden, MC, Vipond, C & Feavers, IM (2012). The effect of iron availability on transcription of the *Neisseria meningitidis fHbp* gene varies among clonal complexes. *Microbiology* **158**: 869-76

Seydel, A, Gounon, P & Pugsley, AP (1999). Testing the '+2 rule' for lipoprotein sorting in the *Escherichia coli* cell envelope with a new genetic selection. *Mol Microbiol* **34**: 810-21

Snape, MD, Pollard, AJ (2005). Meningococcal polysaccharide-protein conjugate vaccines. *Lancet Infect Dis* **5**: 21-30

Southan C, Sharman JL, Benson HE, Faccenda E, Pawson AJ, Alexander SP *et al* (2016). The IUPHAR/BPS Guide to PHARMACOLOGY in 2016: towards curated quantitative interactions between 1300 protein targets and 6000 ligands. *Nucleic Acids Res* doi: 10.1093/nar/gkv1037

Walsh, NP, Alba, BM, Bose, B, Gross, CA & Sauer, RT (2003). OMP peptide signals initiate the envelope-stress response by activating DegS protease via relief of inhibition mediated by its PDZ domain. *Cell* **113**: 61-71

Wilson, MM, Bernstein, HD (2016). Surface-Exposed Lipoproteins: An Emerging Secretion Phenomenon in Gram-Negative Bacteria. *Trends Microbiol* **24**: 198-208

Yakushi, T, Tajima, T, Matsuyama, S & Tokuda, H (1997). Lethality of the covalent linkage between mislocalized major outer membrane lipoprotein and the peptidoglycan of *Escherichia coli*. *J Bacteriol* **179**: 2857-62.

Yongye, AB, Gonzalez-Outeirino, J, Glushka, J, Schultheis, V & Woods, RJ (2008). The conformational properties of methyl alpha-(2,8)-di/trisialosides and their N-acyl analogues: implications for anti-*Neisseria meningitidis* B vaccine design. *Biochemistry* **47**: 12493-514

Zapun A, Morlot C, Taha MK. (2016). Resistance to β-Lactams in Neisseria ssp Due to Chromosomally Encoded Penicillin-Binding Proteins. *Antibiotics* (Basel). **5**: 1-12

Zeng W, Eriksson E, Chua B, Grollo L, Jackson DC. (2010) Structural requirement for the agonist activity of the TLR2 ligand Pam2Cys. *Amino Acids* **39**: 471-80.

Zhang, A, Wassarman, KM, Rosenow, C, Tjaden, BC, Storz, G & Gottesman, S (2003). Global analysis of small RNA and mRNA targets of Hfq. *Mol Microbiol* **50**: 1111-24

Zhang, Q, Li, Y & Tang, CM (2010). The role of the exopolyphosphatase PPX in avoidance by *Neisseria meningitidis* of complement-mediated killing. *J Biol Chem* **285**: 34259-68

Zuckert, WR (2014). Secretion of bacterial lipoproteins: through the cytoplasmic membrane, the periplasm and beyond. *Biochim Biophys Acta* **1843**: 1509-16

Table 1 Drug susceptibilities of MC58, MC58Lnt and MC58LntC.

Drug	Dose(μg/ml)		
	MC58	MC58Lnt	MC58LntC
Tunicamycin[a]	50	50	50
Ceftriaxone[b]	0.00097	0.00049	0.0097
Penicillin G[b]	0.025	0.0125	0.025
Chloramphenicol[b]	2.0	1.0	2.0
Sulfalinamide[c]	100	25	100
Globomycin[c]	6.25	1.5625	6.25
Ciprofloxacin[d]	0.00391	0.00024	0.00391
Rifampicin[e]	0.125	0.00195	0.125

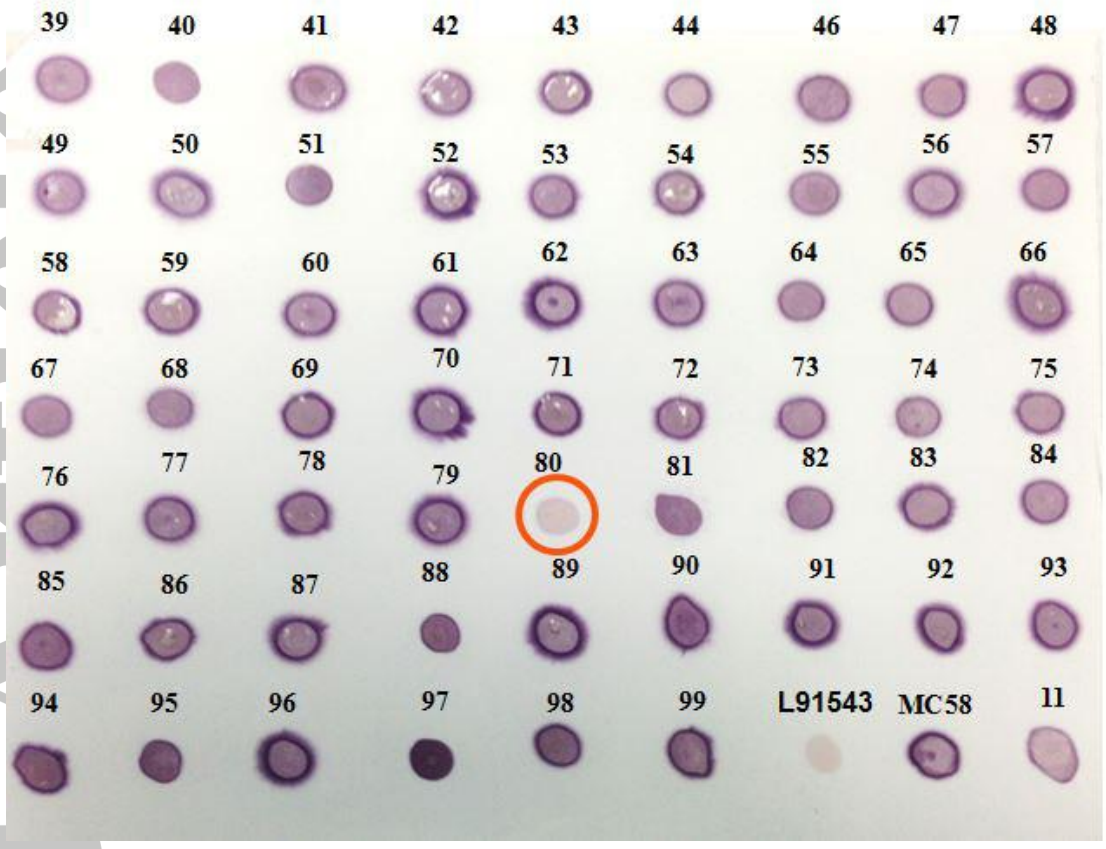

Figure 1 Immuno-dot blot of whole cell suspensions of individual Tn mutants of strain MC58 probed with anti-FHbp antibody, JAR4. Strains MC58 and L91543 were included as positive and negative control strains for reactivity to JAR4.

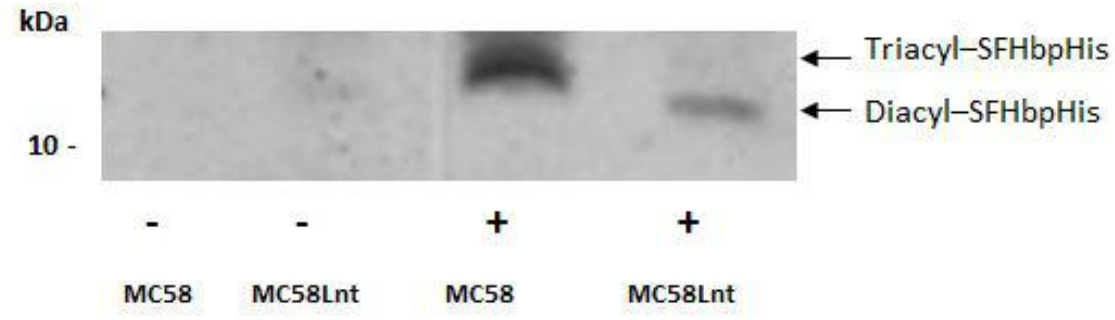

Figure 2. Western immunoblot of whole cell lysates from strains MC58 and MC58Lnt transformed to express His-tagged truncated FHbp, probed with anti-His antibody (lanes 3 and 4 respectively). Lanes 1 and 2 are non-transformed MC58 and MC58Lnt strains. Data are representative of 5 independent experiments.

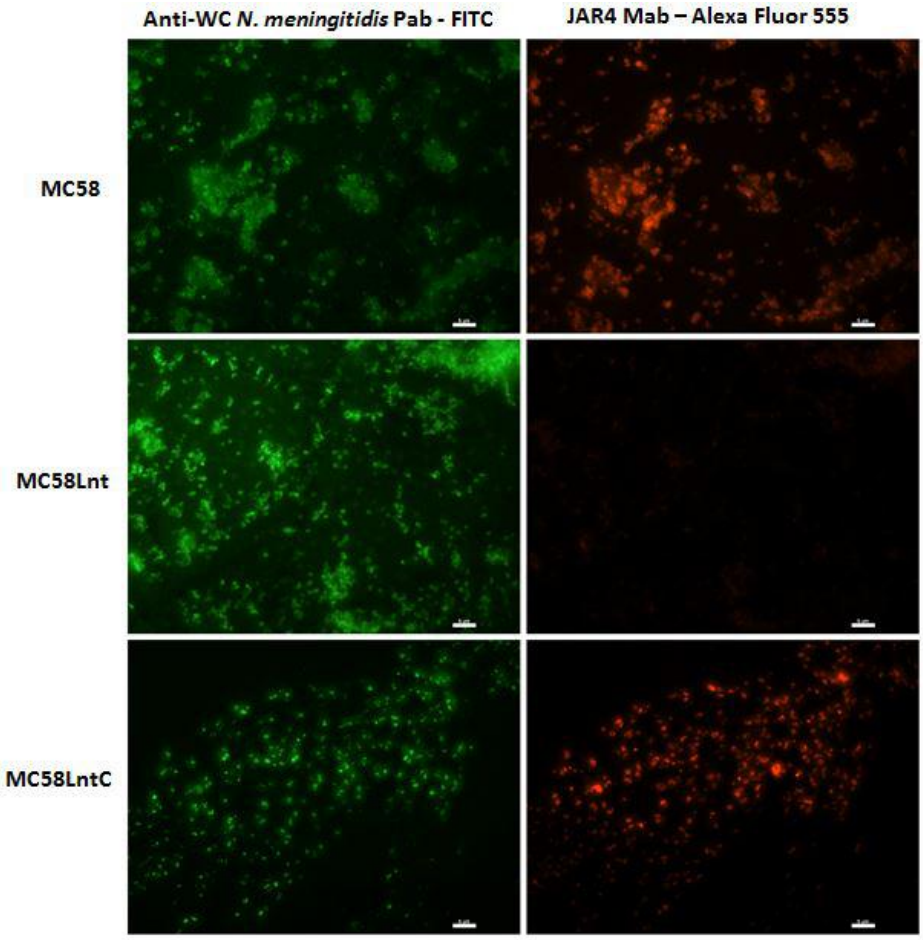

Figure 3. Immunofluorescence microscopy of whole cells of strains MC58, MC58Lnt and MC58LntC. To confirm the presence of meningococcal cells, cells were incubated with FITC-labelled rabbit polyclonal IgG raised against whole cell N. meningitidis (left and right panel). To compare FHbp cell surface expression between the strains, cells were also incubated with anti-FHbp antibody JAR4 which was detected by Alexa Fluor 555 labelled donkey anti-mouse IgG secondary antibody (right panel).

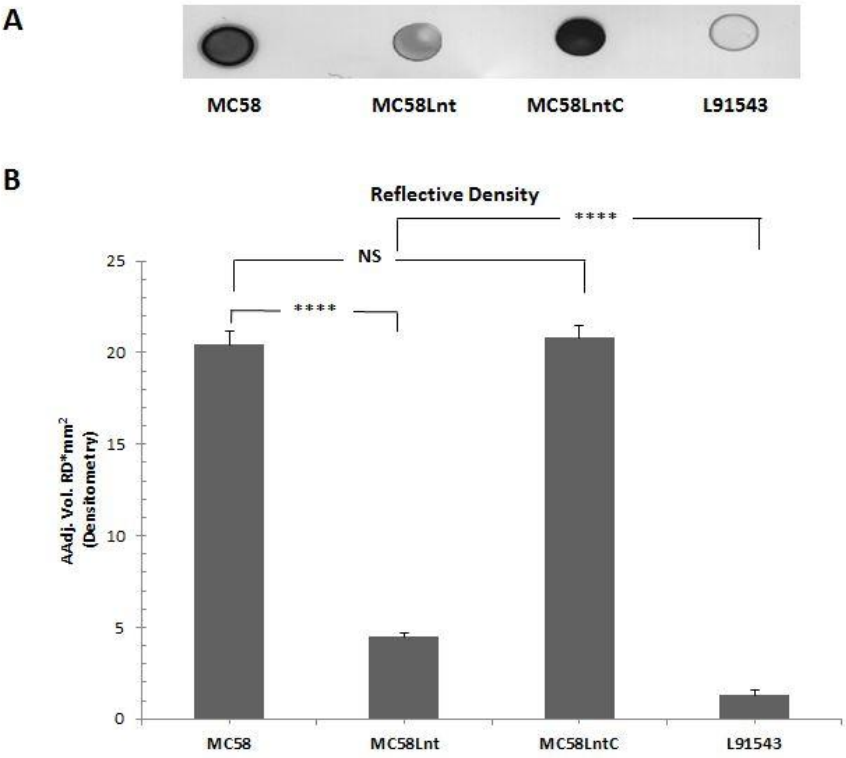

Figure 4. A. The binding of standardised whole cell suspensions of strains MC58, MC58Lnt, MC58LntC and L91543 to anti-FHbp antibody, JAR4, in an immuno-dot blot assay to compare the level of cell surface expression of FHbp between these strains. The image is representative of multiple independent experiments (n=6). B. The reflective density was measured by a GS-800™ calibrated densitometer. All columns represent mean ☐} SEM, ****p≤0.0001. NS Not significant. F achieved p<0.05, no significant variance in homogeneity seen.

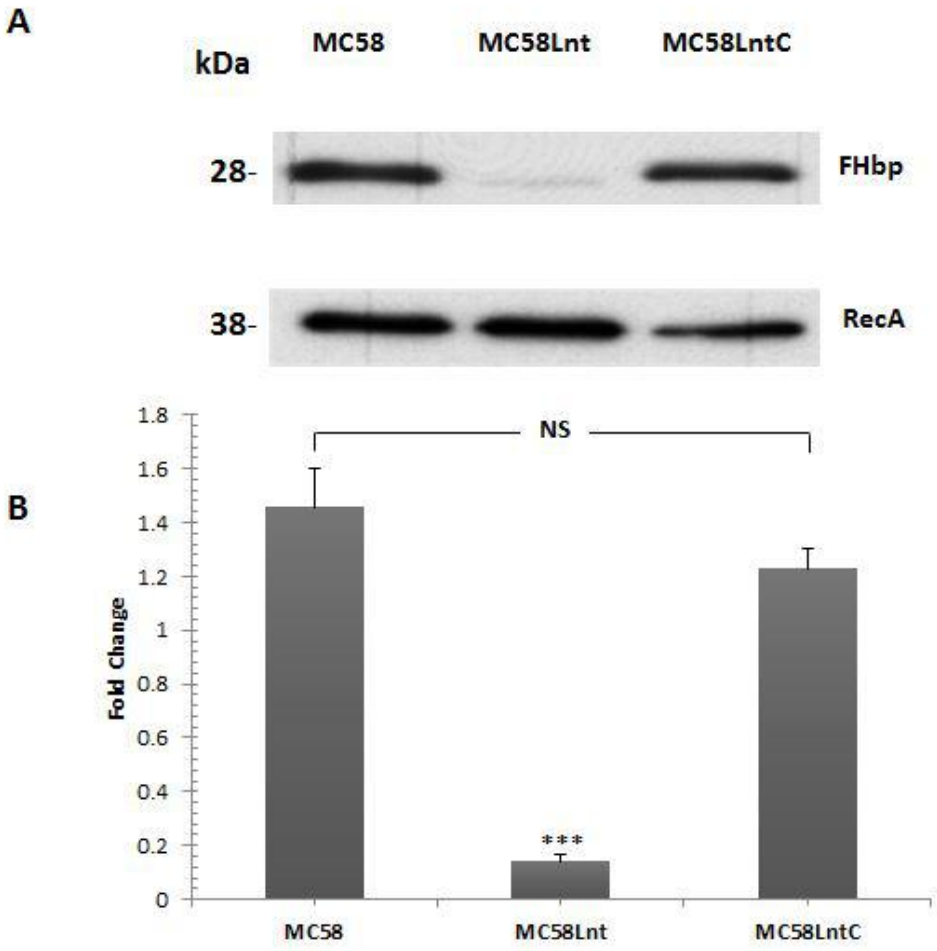

Figure 5. A. Western immunblot of whole cell lysates from strains MC58, MC58Lnt and MC58LntC probed with anti-FHbp antibody, JAR4. Equal protein loading was confirmed by the determination of RecA protein in each sample. The image is representative of multiple independent experiments (n=8). B. The expression of FHbp was determined in MC58, MC58Lnt and MC58LntC by densitometry and normalised to RecA protein. The reflective density was measured by a GS-800™ calibrated densitometer. All columns represent mean ☐} SEM, *p≤0.001 vs strain MC58. NS, Not significant. F achieved $p<0.05$, no significant variance in homogeneity seen.

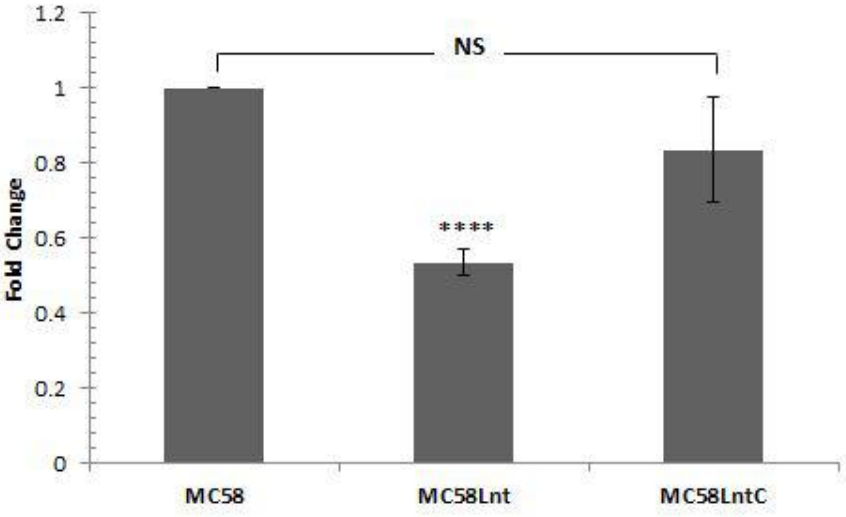

Figure 6 Determination of fHbp mRNA levels in bacterial strains MC58, MC58Lnt and MC58LntC by qRT-PCR analysis. The data was obtained from 6 independent biological replicates (n=6) with each including 2 technical replicates and normalised against the house-keeping gene, recA. All columns represent mean ☐} SEM, ****p≤0.0001 vs strain MC58. NS, Not significant. F achieved p<0.05, no significant variance in homogeneity seen.

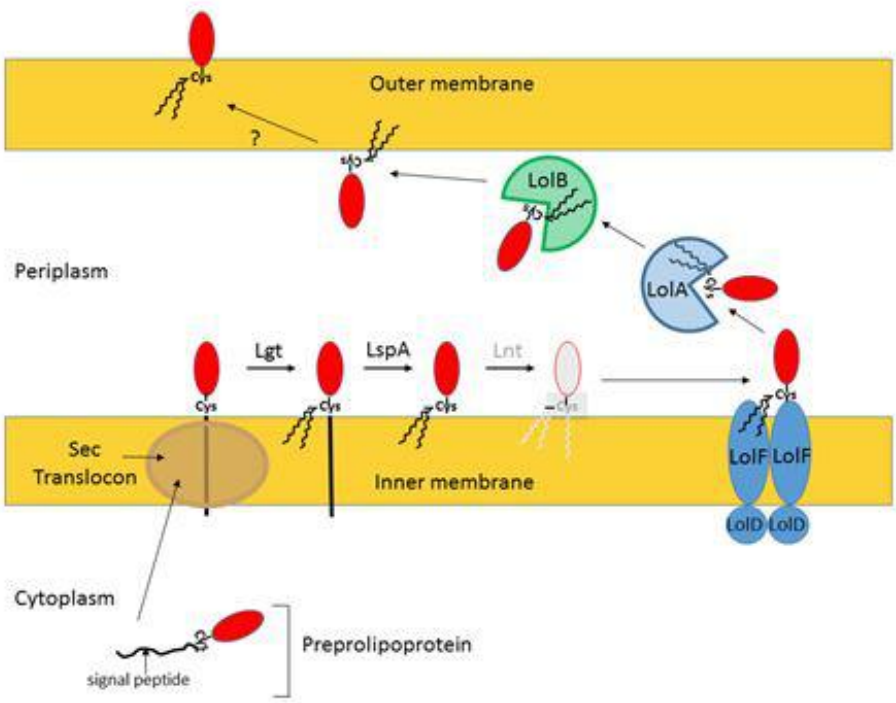

Figure 7 Model for lipoprotein processing and sorting of tri- and diacylated lipoproteins in N. meningitidis. The signal peptide at the N terminus of the preprolipoprotein signals for its translocation across the inner membrane by the Sec apparatus. Once in the periplasm, Lgt adds a diacylglyceride to the conserved cysteine, the last amino acid of the lipobox at the C terminus of the signal peptide. LspA cleaves the signal peptide exposing the diacylated cysteine which becomes the +1 residue to which Lnt adds the third acyl chain. Both the fully mature lipoprotein or the apolipoprotein resulting from mutation of Lnt are recognised by the LolFD transporter complex (LoVullo et al., 2015). The LolA chaperone receives the lipoprotein or apolipoprotein and delivers it to the outer membrane anchored lipoprotein LolB which inserts it into the outer membrane. The mechanism of transport across the outer membrane for export to the cell surface remains to be investigated (Wilson and Bernstein, 2015).